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APPLICATION NUMBER: 60/485,463
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Express Mail Label No.: EV 329 478 974 US

PATENT
Attorney Docket No.: KNAUTHE-08190**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

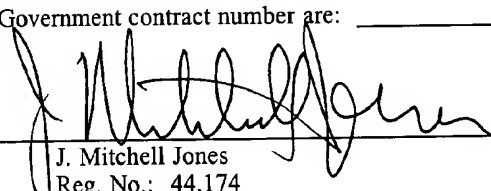
This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

Docket Number		KNAUTHE-08190		Type a plus sign (+) inside this box →	14 72 U.S. PTO 0/485463 07/08/03
INVENTOR(s) / APPLICANT(s)					
Last Name	First Name	Middle Initial	Residence (City and Either State or Foreign Country)		
Kolossov Kettenhofen Kopp Bohlen Schwengberg	Eugen Ralf Isabella Heribert Silke		Köln, Germany Bonn, Germany Köln, Germany Köln, Germany Düren, Germany		
TITLE OF THE INVENTION (280 Characters Max.)					
Novel Method For The Preparation Of Embryoid Bodies (EBs) And Uses Thereof					
CORRESPONDENCE ADDRESS					
MEDLEN & CARROLL, LLP 101 Howard Street, Suite 350 San Francisco, California 94105 United States of America					
ENCLOSED APPLICATION PARTS (Check All That Apply)					
<input checked="" type="checkbox"/> Specification	Number of Pages	27	<input type="checkbox"/> Small Entity Statement		
<input checked="" type="checkbox"/> Drawing(s)	Number of Sheets	0	<input type="checkbox"/> Other (Specify): Power of Attorney		
			<input type="checkbox"/> Other (Specify): Assignment		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input type="checkbox"/> Charge Account No. 08-1290 in the amount of \$80.00. An originally executed duplicate of this transmittal is enclosed for this purpose.		FILING FEE AMOUNT (\$)		\$80.00	
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any deficiency in the payment of the required fee(s), and/or credit any overpayment, to Deposit Account No.: 08-1290. An originally executed duplicate of this transmittal is enclosed for this purpose.					

This invention was made by an agency of the United States Government under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

Date: July 8, 2003
J. Mitchell Jones
Reg. No.: 44,174
MEDLEN & CARROLL, LLP
101 Howard Street, Suite 350
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(608) 218-6900☐ Additional inventors are being named on separately numbered sheets attached hereto.

Express Mail Label No.: EV 329 478 974 US

PATENT
Attorney Docket No.: KNAUTHE-08190

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kolossov, *et al.*

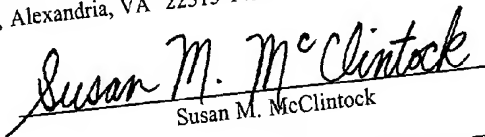
For: Novel Method For The Preparation Of Embryoid Bodies (EBs)
And Uses Thereof

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450



CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on July 8, 2003, in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. § 1.10, Mailing Label Number EV 329 478 974 US, addressed to: Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.


Susan M. McClintock

TRANSMITTAL COVER SHEET FOR FILING PROVISIONAL APPLICATION
(37 C.F.R. § 1.51(2)(i))

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

1. The following comprises the information required by 37 C.F.R. § 1.51(a)(2)(i)(A):
2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(a)(2)(i)(B)):

Eugen Kolossov
Ralf Kettenhofen
Isabella Kopp
Heribert Bohlen
Silke Schwengberg

3. Address(es) of the inventor(s), as numbered above (37 C.F.R. § 1.51(a)(2)(i)(C)):

Franz-Hitze-Str. 1, 50672 Köln, Germany
Ubierstr. 3, 53173 Bonn, Germany
Herbesthalerstr. 3, 50933 Köln, Germany
Auerstrasse 4, 50733 Köln, Germany
Dampfmühlenstr. 107, 52355 Düren, Germany

4. The title of the invention is (37 C.F.R. § 1.51(a)(2)(i)(D)):
Novel Method For The Preparation Of Embryoid Bodies (EBs) and Uses Thereof

Express Mail Label No.: EV 329 478 974 US

5. The name, registration, and telephone number of the attorney (if applicable) is (37 C.F.R. § 1.51(a)(2)(i)(E)):

J. Mitchell Jones
Reg. No.: 44,174
Tel.: (608) 218-6900

(complete the following, if applicable)

___ A Power of Attorney accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(a)(2)(i)(F)):

Docket No.: KNAUTHE-08190

7. The correspondence address for this application is (37 C.F.R. § 1.51(a)(2)(i)(G)):

MEDLEN & CARROLL, LLP
101 Howard Street, Suite 350
San Francisco, California 94105

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(a)(2)(i)(H)):

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

X No.

___ Yes.

The name of the U.S. Government agency and the Government contract number are: _____.

9. Identification of documents accompanying this cover sheet:

- A. Documents required by 37 C.F.R. § 1.51(a)(2)(ii)-(iii):

Specification: No. of pages **27**

Drawings: No. of sheets **0**

- B. Additional documents:

X Claims: No. of claims **25**

___ Power of Attorney

___ Small Entity Statement

___ Assignment

___ Other

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10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is \$160.00 for other than a small entity, and \$80.00 for a small entity.

X Applicant is a small entity.

11. Small Entity Statement

— The verified statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is(are) attached.

12. Fee payment being made at this time

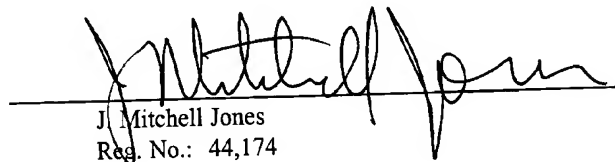
— Charge Deposit Account No. 08-1290 in the amount of \$80.00. A duplicate of this transmittal is enclosed for this purpose.

13. Method of Fee Payment:

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Date: July 8, 2003

J. Mitchell Jones
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Novel method for the preparation of Embryoid Bodies (EBs) and uses thereof

Field of the invention

5 The present invention relates to a method for producing embryoid bodies (EBs) from multi- or pluripotent cells. In particular, the instant invention concerns a method of generating embryoid bodies (EBs) comprising agitation of a liquid suspension culture of multi- or pluripotent cells in a container at a concentration of about 1 to 5×10^6 cells/ml until generation of cell aggregates, diluting the suspension and agitation of the suspension until formation of
10 EBs. Furthermore, the present invention relates to the use of the method and EBs so obtained for a variety of applications including but not limited to "loss of function" assays with ES cells containing homozygous mutations of specific genes, "gain of function" assays with ES cells overexpressing exogenous genes, developmental analysis of teratogenic/embryotoxic compounds in vitro, pharmacological assays and the establishment of model systems for
15 pathological cell functions, and application of differentiation and growth factors for induction of selectively differentiated cells which can be used as a source for tissue grafts.

Background art

Precursor cells have become a central interest in medical research. On the one hand precursors
20 can replace cells that are senescent or damaged by injury or disease and on the other hand these cells represent an ideal model for studying development and differentiation and the factors influencing these processes. Employing conventional cell lines for these studies has the disadvantage that individual cell lines may not be fully representative of the complex biology of an intact organism. Moreover, even repeating the tests in multiple cell lines does
25 not reproduce or account for the complex interactions among cells and tissues that occurs in an organism.

Efforts have been made for a couple of years to employ permanent cultures of totipotent/pluripotent embryonic stem (ES) cells for the detection of embryotoxic and mutagenic substances and for the preparation of tissue grafts. ES cells can differentiate in
30 vitro in embryo-like aggregates, so-called embryoid bodies (EBs), derivatives of all three germ layers, i.e. mesoderm, ectoderm and endoderm. Thus, embryoid bodies are particularly suited for teratogenic/embryotoxicological studies as well as identification of cell type and tissue promoting factors, and as precursors for implant tissue for the treatment of damaged

organs such as infarcted heart. Several protocols for the in vitro production of EBs have been described.

For example, WO02/051987 describes a protocol to obtain embryoid bodies in which the manufacturing takes place preferably with the “hanging drop” method or by methylcellulose culture (Wobus et al., Differentiation 48 (1991), 172-182).

Alternatively to this, spinner flasks (stirring cultures) are described as culture method. Therefor, the undifferentiated ES-cells are introduced into stirring cultures and are mixed permanently according to an established procedure. Therefor, 10 million ES-cells are introduced into 150 ml medium with 20 % FCS and are stirred constantly with the rate of 20 rpm., wherein the direction of the stirring motion is changed regularly. 24 hours after introduction of the ES-cells an extra 100 ml medium with serum is added and thereupon 100 - 150 ml of the medium is exchanged every day (Wartenberg et al., FASEB J. 15 (2001), 995-1005). Under these culture conditions large amounts of ES-cell-derived cells, i.e. cardiomyocytes, endothelial cells, neurons etc. depending on the composition of the medium may be obtained. The cells are selected by means of the resistance gene either still within the stirring culture or after plating, respectively. However, those methods are cumbersome and/or do not provide sufficient amounts of embryoid bodies suitable for example for High Throughput Screening (HTS) assays.

Thus, there remains a need for reliable, easy and cost-effective methods which are capable of providing EBs in sufficient quality and quantity. The solution to this technical problem is achieved by providing the embodiments characterized in the claims, and described further below.

Summary of the invention

The present invention relates to a method for producing embryoid bodies (EBs) from multi- or pluripotent cells comprising

- (a) agitation of a liquid suspension culture of multi- or pluripotent cells in a container at a concentration of about 1 to 5×10^6 cells/ml until generation of cell aggregates; and
- (b) diluting the suspension and agitation of the suspension until formation of EBs.

The present invention also concerns the embryoid bodies obtained by the described method of the present invention as well as the differentiated cell or tissue derived from such embryoid bodies, in particular cardiomyocytes.

Furthermore, the present invention relates to the use of the method, embryoid bodies, cells and tissue of the present invention for “loss of function” assays of specific genes, “gain of function assays” of exogenous genes, developmental analysis of teratogenic/embryotoxic compounds, pharmacological assays, microarray systems, establishment of model systems for pathological cell functions, and application of differentiation and growth factors for induction of selectively differentiated cells or as a source for tissue grafts.

In addition, the instant invention relates to a kit for use in a method of the invention comprising culture media components, selectable markers, reference samples, microarrays, vectors, probes, containers, multi- or pluripotent cells.

Moreover, the present invention is directed to the use of cell containers, devices for agitation and/or culturing cells, culture media and components thereof, multi- or pluripotent cells, vectors, and microarrays for a method of the present invention.

In particular, the present invention relates to test systems to identify substances that influence the differentiation of cells into certain cell types. Therefore, the present invention provides a method for obtaining and/or profiling a modulator of cell differentiation. This method comprises contacting a test sample containing EBs obtained by the method of the present invention with the substance to be tested; and then determining the effect of the test substance on the EBs or on the amount of the reporter gene product or activity compared to a control sample. The EBs production and test system provided by the present invention is useful for drug screening purposes.

Definitions

For the purposes of this description, the term "stem cell" can refer to either stem cell or germ cell, for example embryonic stem (ES) and germ (EG), respectively. Minimally, a stem cell has the ability to proliferate and form cells of more than one different phenotype, and is also capable of self renewal-either as part of the same culture, or when cultured under different conditions. Embryonic stem cells are also typically telomerase positive and OCT-4 positive. Telomerase activity can be determined using TRAP activity assay (Kim et al., Science 266 (1997), 2011), using a commercially available kit (TRAPeze(R) XK Telomerase Detection Kit, Cat. s7707; Intergen Co., Purchase N.Y.; or TeloTAGGG(TM) Telomerase PCR

ELISApplus, Cat. 2,013,89; Roche Diagnostics, Indianapolis). hTERT expression can also be evaluated at the mRNA level by RT-PCR. The LightCycler TeloTAGGG(TM) hTERT quantification kit (Cat. 3,012,344; Roche Diagnostics) is available commercially for research purposes.

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In accordance with the present invention, the term embryonic stem (ES) cell includes any multi- or pluripotent stem cell derived from pre-embryonic, embryonic, or fetal tissue at any time after fertilization, and have the characteristic of being capable under appropriate conditions of producing progeny of several different cell types that are derivatives of all of the three germinal layers (endoderm, mesoderm, and ectoderm), according to a standard art-accepted test, such as the ability to form a teratoma in 8-12 week old SCID mice.

"Embryonic germ cells" or "EG cells" are cells derived from primordial germ cells. The term "embryonic germ cell" is used to describe cells of the present invention that exhibit an embryonic pluripotent cell phenotype. The terms "human embryonic germ cell (EG)" or "embryonic germ cell" can be used interchangeably herein to describe mammalian, preferably human cells, or cell lines thereof, of the present invention that exhibit a pluripotent embryonic stem cell phenotype as defined herein. Thus, EG cells are capable of differentiation into cells of ectodermal, endodermal, and mesodermal germ layers. EG cells can also be characterized by the presence or absence of markers associated with specific epitope sites identified by the binding of particular antibodies and the absence of certain markers as identified by the lack of binding of certain antibodies.

"Pluripotent" refers to cells that retain the developmental potential to differentiate into a wide range of cell lineages including the germ line. The terms "embryonic stem cell phenotype" and "embryonic stem-like cell" also are used interchangeably herein to describe cells that are undifferentiated and thus are pluripotent cells.

Included in the definition of ES cells are embryonic cells of various types, exemplified by human embryonic stem cells, described by Thomson et al. (Science 282 (1998), 1145); embryonic stem cells from other primates, such as Rhesus stem cells (Thomson et al., Proc. Natl. Acad. Sci. USA 92 (1995), 7844), marmoset stem cells (Thomson et al., Biol. Reprod. 55 (1996), 254) and human embryonic germ (hEG) cells (Shamblott et al., Proc. Natl. Acad. Sci. USA 95 (1998), 13726). Other types of pluripotent cells are also included in the term. Any cells of mammal origin that are capable of producing progeny that are derivatives of all

three germinal layers are included, regardless of whether they were derived from embryonic tissue, fetal tissue, or other sources. The stem cells employed in accordance with the present invention that are preferably (but not always necessary) karyotypically normal. However, it is preferred not to use ES cells that are derived from a malignant source.

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"Feeder cells" or "feeders" are terms used to describe cells of one type that are co-cultured with cells of another type, to provide an environment in which the cells of the second type can grow. The feeder cells are optionally from a different species as the cells they are supporting. For example, certain types of ES cells can be supported by primary mouse embryonic fibroblasts, immortalized mouse embryonic fibroblasts (such as murine STO cells, e.g., Martin and Evans, Proc. Natl. Acad. Sci USA 72 (1975), 1441-1445), or human fibroblast-like cells differentiated from human ES cells, as described later in this disclosure. The term "STO cell" refers to embryonic fibroblast mouse cells such as are commercially available and include those deposited as ATCC CRL 1503.

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The term "embryoid bodies" (EBs) is a term of art synonymous with "aggregate bodies". The terms refer to aggregates of differentiated and undifferentiated cells that appear when ES cells overgrow in monolayer cultures, or are maintained in suspension cultures. Embryoid bodies are a mixture of different cell types, typically from several germ layers, distinguishable by morphological criteria; see also *infra*. As used herein, "embryoid body", "EB" or "EB cells" typically refers to a morphological structure comprised of a population of cells, the majority of which are derived from embryonic stem (ES) cells that have undergone differentiation. Under culture conditions suitable for EB formation (e.g., the removal of Leukemia inhibitory factor or other, similar blocking factors), ES cells proliferate and form small mass of cells that begin to differentiate. In the first phase of differentiation, usually corresponding to about days 1-4 of differentiation for humans, the small mass of cells forms a layer of endodermal cells on the outer layer, and is considered a "simple embryoid body". In the second phase, usually corresponding to about days 3-20 post-differentiation for humans, "complex embryoid bodies" are formed, which are characterized by extensive differentiation of ectodermal and mesodermal cells and derivative tissues. As used herein, the term "embryoid body" or "EB" encompasses both simple and complex embryoid bodies unless otherwise required by context. The determination of when embryoid bodies have formed in a culture of ES cells is routinely made by persons of skill in the art by, for example, visual inspection of the morphology. Floating masses of about 20 cells or more are considered to be embryoid bodies; see, e.g.,

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Schmitt et al., *Genes Dev.* 5 (1991), 728-740; Doetschman et al. *J. Embryol. Exp. Morph.* 87 (1985), 27-45. It is also understood that the term "embryoid body", "EB", or "EB cells" as used herein encompasses a population of cells, the majority of which being pluripotent cells capable of developing into different cellular lineages when cultured under appropriate conditions. As used herein, the term also refers to equivalent structures derived from primordial germ cells, which are primitive cells extracted from embryonic gonadal regions; see, e.g., Shambloott, et al. (1998), *supra*. Primordial germ cells, sometimes also referred to in the art as EG cells or embryonic germ cells, when treated with appropriate factors form pluripotent ES cells from which embryoid bodies can be derived; see, e.g., US Patent US-A-5,670,372; Shambloott, et al., *supra*.

If not stated otherwise the terms "compound", "substance" and "(chemical) composition" are used interchangeably herein and include but are not limited to therapeutic agents (or potential therapeutic agents), agents of known toxicities such as neurotoxins, hepatic toxins, toxins of hematopoietic cells, myotoxins, carcinogens, teratogens, or toxins to one or more reproductive organs. The chemical compositions can further be agricultural chemicals, such as pesticides, fungicides, nematocides, and fertilizers, cosmetics, including so-called "cosmeceuticals", industrial wastes or by-products, or environmental contaminants. They can also be animal therapeutics or potential animal therapeutics.

Test substances that can be tested with the methods of the present invention comprise all kinds of chemicals, for example textile chemicals, laboratory chemicals, industrial chemicals, medical chemicals, printing chemicals, leather chemicals, in particular household products including bleaches, toilet, blocks, washing-up liquids, soap powders and liquids, fabric conditioners, window, oven, floor, bathroom, kitchen and carpet cleaners, dishwasher detergents and rinse aids, watersoftening agents, descalers, stain removers, polishes, paints, paint removers, lubricant, dyestuff, coating, glues, solvents, varnishes, air fresheners, moth balls insecticides and the like.

New ingredients for household products are constantly being developed and needed to be tested. For example, in recent years new enzymes (to digest stains) and "optical brighteners" (which make washing appear whiter) have been developed for use in washing powders and liquids. New surfactants (which cut through grease to remove ingrained dirt) and chemical "builders" (which act as water softeners and enable surfactants to work more effectively) have been developed for use in washing powders and liquids, washing-up liquids and various cleaning agents. But also medical materials have to be tested, for example dental materials

such as new filling polymers, metal alloys, and bioactive ceramic. Furthermore, chemical compositions of any part of a device, such as an electrode, adhesives, paste, gel or cream including the concentrations of the different ingredients and impurities present may be tested with the method of the present invention.

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Detailed description

Stem cells of various kinds have become an extremely attractive modality in regenerative medicine. They can be proliferated in culture, and then differentiated in vitro or in situ into the cell types needed. This plasticity makes them ideal models for toxicity testing. Particularly
10 embryoid bodies (EBs) which consist of different cell types of the three germ layers that interact with each other provide a highly sensitive test system. In one embodiment, the cells within an embryoid bodies are substantially synchronized for their differentiation. Accordingly, at known intervals, the majority of the synchronized cells differentiate into the three embryonic germ layers and further differentiate into multiple tissue types, such as
15 cartilage, bone, smooth and striated muscle, and neural tissue, including embryonic ganglia. Thus, the cells within embryoid bodies provide a much closer model to the complexity of whole organisms than do traditional single cell or yeast assays, while still avoiding the cost and difficulties associated with the use of mice and larger mammals. Moreover, the recent availability of human embryoid bodies improves the predictive abilities of the invention by
20 providing an even closer vehicle for modeling toxicity in human organ systems, and in humans. Thus, the provision of EBs in sufficient quantity and in an economically efficient manner is the main object of this invention.

Accordingly, the present invention relates to a method for producing embryoid bodies (EBs)
25 from multi- or pluripotent cells comprising
(a) agitation of a liquid suspension culture of multi- or pluripotent cells in a container at a concentration of about 1 to 5×10^6 cells/ml until generation of cell aggregates; and
(b) diluting the suspension and agitation of the suspension until formation of EBs.

The present invention is based on agitation technique rather than EBs stirring or hanging drop
30 cultures previously described as the method of choice.

In accordance with the present invention, it has surprisingly been found that embryonic stem (ES) cell aggregates, so called Embryoid Bodies (EBs) can be generated in large amounts and high density, which in turn can be induced to differentiate to particular cell types and tissue such as cardiomyocytes, neurones, endothelial cells and the like. The present invention is based

on the observation that agitation of a certain amount and concentration of ES cells in an appropriate container is superior to, for example, the stirring culture method for the preparation of ES cell aggregates, in particular embryoid bodies. With the method of the invention the yield of embryoid bodies can be considerably be improved compared to conventional methods, since the cell aggregates can be cultured in large volumes and higher density than in the previous methods. The method of the invention also allows generating tissue in sufficient amounts for therapeutic uses, wherein the target tissue can be purified according to standard methods such as described in WO02/051987. Accordingly, the method of the present invention provides several advantages over the prior art methods for the preparation of embryoid bodies.

First, the embryoid bodies, and thus any desired precursors and cell types are provided in large amounts and high density and allows compound screening on industrial scale.

Second, the method of the invention is quite easy to perform contrary to, for example, the hanging drop method. In accordance with this, the method of the invention is much more reliable and reproducible than the classical methods.

Third, the operating expense for large-scale production of embryoid bodies in accordance with the method of the present invention is also quite low compared to other fermenter cultures.

Fourth, compared to cultures in spinner flasks, in the method of the invention the ES cells are much less exposed to shear stress, whereby the capability of the cells to differentiate in an appropriate manner is not negatively influenced.

Fifth, the preparation of large amounts of ES cells aggregates and tissue derived therefrom, respectively, under identical conditions ("batch") is important, e.g., for toxicological and pharmacological investigations and for the generation of tissue for transplantation purposes.

As described in the examples, the method of the present invention can be generally described as follows:

1. Optionally conventional culturing of ES cells on feeder cells, for example, mouse embryonic fibroblasts;
2. Preparation of a cell suspension with a density of about 1 to 5×10^6 ES cells/ml, preferably 1.5 to 2.5×10^6 ES cells/ml, most preferably about 2×10^6 ES cells/ml and transfer in an appropriate container such as petri dish;
3. Agitating the suspension for about six hours on a rocking table at about 50rpm until generation of cell aggregates;

4. Dilution of the suspension 1:10 and transfer into an appropriate second container such as preferably T25 flasks;
5. Agitating the suspension for about 16 to 20, preferably 18 hours on the rocking table; optionally
- 5 6. Dividing cell aggregates to the final and desired concentration; optionally
7. Differentiating the cells into the desired tissue; optionally
8. Selection of desired differentiated cell types and tissue with the help of preferably resistance markers, for example puromycin selection; and optionally
9. Use of the embryoid bodies, cells or tissue for a variety of in vitro tests or for
- 10 therapeutic uses such as transplantation.

The invention can be practiced using stem cells of any vertebrate species. Included are stem cells from humans; as well as non-human primates, domestic animals, livestock, and other non-human mammal. Amongst the stem cells suitable for use in this invention are primate

15 pluripotent stem cells derived from tissue formed after gestation, such as a blastocyst, or fetal or embryonic tissue taken any time during gestation. Non-limiting examples are primary cultures or established lines of embryonic stem cells. The invention is also applicable to adult stem cells. It is referred to the literature of Anderson et al., Nat. Med. 7 (2001), 393 395 and Anderson et al., 2001, Gage, F.H., 200 and Prockop, Science 276 (1997), 71 74, wherein the

20 extraction and culture of those cells is described. Thus, said multi- or pluripotent cells used in accordance with the method of the present invention are usually embryonic stem (ES) cells, primordial germ (EG) cells or adult stem cells, most preferably ES cells.

As mentioned before, several sources for ES cells are at the disposal of the skilled person of

25 which human stem cells are preferred for most of the embodiments of the present invention, in particular for therapeutic purposes such as transplantation. Human embryonic stem cells and their use for preparing different cell and tissue types are also described in Reprod. Biomed. Online 4 (2002), 58-63. Embryonic stem cells can be isolated from blastocysts of members of the primate species (Thomson et al., Proc. Natl. Acad. Sci. USA 92 (1995),

30 7844). Human Embryonic Germ (EG) cells can be prepared from primordial germ cells present in human fetal material taken about 8-11 weeks after the last menstrual period. Suitable preparation methods are described in Shamblott et al., Proc. Natl. Acad. Sci. USA 95 (1998), 13726. Method for making cells that resemble embryonic stem cells or embryonic germ cells in morphology and pluripotency derived from primordial germ cells isolated from

human embryonic tissue, such as from the gonadal ridges of human embryo, are described in US patent US-A-6,245,566.

Recently, it has been reported that exfoliated human deciduous tooth, a comparable very accessible tissue, contains multipotent stem cells that were identified to be a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neural cells, adipocytes, and odontoblasts; see Miura et al., Proc. Natl. Acad. Sci. USA, (2003), Apr 25; p S0027-8424. After in vivo transplantation, those cells were found to be able to induce bone formation, generate dentin, and survive in mouse brain along with expression of neural markers. Furthermore, multilineage potential of homozygous stem cells derived from metaphase II oocytes has been described in by Lin et al. in Stem Cells 21 (2003), 152-161. Various sources of precursor cells in postnatal muscles and the factors that may enhance stem cell participation in the formation of new skeletal and cardiac muscle in vivo are reviewed in Grounds et al. J. Histochem. Cytochem. 50 (2002), 589-610. Purification of rare Hematopoietic Stem Cell(s) (HSC) to homogeneity that home to bone marrow is described in US2003/0032185. These adult bone marrow cells are described to have tremendous differentiative capacity as they can also differentiate into epithelial cells of the liver, lung, GI tract, and skin. This finding may contribute to clinical treatment of genetic disease or tissue repair. Furthermore, techniques such as nuclear transfer for embryo reconstruction may be employed wherein diploid donor nuclei are transplanted into enucleated MII oocytes. This technology along with other procedures that aid in the establishment of customized embryonic stem (ES) cell lines that are genetically identical to those of the recipient have been reviewed by Colman and Kind, Trends Biotechnol 18 (2000), 192-196. In order to avoid graft rejection associated with allogenic or xenogenic cells in transplantation syngenic or autologous cells and recipients are preferably used in the corresponding embodiments of the invention. In view of the recent discovered sources of stem cells such as from the bone marrow and tooth it should be possible to accomplish this demand without the need to resort to embryonic cells and tissue. Alternatively, cells may be genetically manipulated to suppress relevant transplantation antigens, see also *infra*, immunosuppressive agents may be used.

The field of stem cell technology is being reviewed by Kiessling and Anderson, Harvard Medical School, in Human Embryonic Stem Cells: An Introduction to the Science and Therapeutic Potential; (2003) Jones and Bartlett Publishers; ISBN: 076372341X.

In order to avoid the use of for example human embryos as the donor for stem cells, which however seems to be justifiable at least under certain circumstances, it may even be possible to employ transgenic non-human animals, in particular mammals as source for embryonic stem cells. For example, compositions and methods for making transgenic swines to be used as xenograft donors is described in US patent 5,523,226. Likewise, WO97/12035 describes methods of producing transgenic animals for xenotransplantation. Furthermore, immunologically compatible animal tissue, suitable for xenotransplantation into human patients, is described in WO01/88096. Method for making embryonic germ cells from porcine are described for example in US patent US-A-6,545,199.

Stem cells can be propagated continuously in culture, using a combination of culture conditions that promote proliferation without promoting differentiation. Traditionally, stem cells are cultured on a layer of feeder cells, typically fibroblast type cells, often derived from embryonic or fetal tissue. The cell lines are plated to near confluence, usually irradiated to prevent proliferation, and then used to support when cultured in medium conditioned by certain cells (e.g. Koopman and Cotton, *Exp. Cell* 154 (1984), 233-242; Smith and Hooper, *Devel. Biol.* 121 (1987), 1-91), or by the exogenous addition of leukemia inhibitory factor (LIF). Such cells can be grown relatively indefinitely using the appropriate culture conditions. In a preferred embodiment of the method of the invention, the cells are cultured on embryonic mouse fibroblasts prior to step (a); see also *supra*.

In principle, any conventional culture medium can be used in the methods of the present invention such as media for isolating and propagating stem cells that can have any of several different formulas, as long as the cells obtained have the desired characteristics, and can be propagated further. Suitable sources include Iscove's modified Dulbecco's medium (IMDM), Gibco, #12440-053; Dulbecco's modified Eagles medium (DMEM), Gibco #11965-092; Knockout Dulbecco's modified Eagles medium (KO DMEM), Gibco #10829-018; 200 mM L-glutamine, Gibco # 15039-027; non-essential amino acid solution, Gibco 11140-050; [beta]-mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029. Exemplary serum-containing ES medium and conditions for culturing stem cells are known, and can be optimized appropriately according to the cell type. Media and culture techniques for particular cell types referred to in the previous section are provided in the references cited herein.

However, preferred is the use of IMDM with 20 % FCS at CO₂ of 5 % while DMEM with 20 % FCS at 7 % CO₂ can also be used but is less preferred. Thus, in a particularly preferred embodiment of the method of the present invention, the culture medium in step (a) and/or in step (b) is IMDM 20% FCS and 5% CO₂.

- 5 Other culture conditions can be adjusted according to standard methods known to the person skilled in the art. Particularly preferred, however, is to perform the method of the present invention, wherein the culture conditions in step (a) and/or (b), most preferably in both steps and during the complete culture period, comprise 37°C and 95% humidity.
- 10 In a particularly preferred embodiment, the method of the invention is performed, wherein the suspension in step (a) is cultured for about 6 hours and/or in step (b) for about 18 hours. In some embodiments, the time for the first culture step of about six hours should be kept as close as possible, the time for the second culture step, i.e. step (b) may vary for example from about 16 to 20 hours. Of course, the person skilled in the art may vary one or more parameters
- 15 indicated herein for the method of the present invention while still working along the gist of the invention that is the use of a liquid suspension cultures of multi- or pluripotent cells being constantly agitated during a defined period of time including a transfer of the cell suspension until cell aggregates have been generated.
- 20 The container to be used in step (a) and (b) can be of any conventional type used in cell culture systems and can be of any appropriate material such as glass or preferably plastic. With respect to culturing cell suspension in step (a) round containers such as petri dishes are preferred. Without intending to be bound by theory it has been observed in experiments performed in accordance with the present invention that the shape of the container may have
- 25 some influence on the yield and the status of the cells and cell aggregates, respectively. It has thus been found that for the first culture step of the liquid suspension culture of multi-or pluripotent cells round containers such as petri dishes are preferably used. In this respect, a ratio of for example 4 ml of cell suspension to 6 cm (diameter) petri dish was found to give very good results. Therefore, the container in step (a) should be preferably chosen such that a
- 30 corresponding ratio of cell suspension and culture surface of the container as described in the examples is achieved.

Similarly, in step (b) the container, for example, culture flask, should preferably be dimensioned as described in the examples for the T25 flasks. Furthermore, in accordance with the

dimension of the containers used in the method of the present invention, the rate of agitation should be adjusted accordingly. Typically, the agitation in step (a) and/or (b) is performed at 50rpm. However, different rates for containers deviating from those used in the examples may be used as well.

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Thus, in a further preferred embodiment of the present invention, the incubation step is conducted in a container made of plastic, and wherein the suspension culture in step (a) is present in a petri dish and/or in step (b) in a culture flask, beaker or tumbler, most preferably in T25 flasks, preferably with agitation at 50rpm. The agitation in step (a) and/or (b) is preferably a horizontal agitation. However, other agitation procedures such as tumbling may be used as well while however less preferred.

After step (a) the cell suspension, i.e. cell aggregates are diluted by a factor of about 10. The actual factor may vary due to, for example, the initial concentration of multi- or pluripotent cells. Generally, the dilution in step (b) is preferably at least about 1:5, more preferably about 1:10 or higher, for example 2 ml of the suspension obtained in step (a) with 18 ml new medium such as IMDM 20% FCS; see also the examples. Most preferably, the dilution factor is 1:10 in case the initial concentration of multi- or pluripotent cells compares to 2×10^6 cells/ml.

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In previous methods for the production of embryoid bodies the yield of embryoid bodies was in a range of 50/ml. With the method of the present invention, however, final concentrations of EBs in a suspension culture of step (b) are possible in a range of 100 to of about more than 1.000/ml, generally in a range of about 500/ml. Preferably, the method is performed such that the culture reaches a concentration of about 500/ml EBs. Thus, even with an experimental set up for the production of embryoid bodies in accordance with the present invention about 10.000 embryoid bodies can be generated in one experiment (starting with ES cells from one 6 cm petri dish) which allows the performance of various tests concomitantly and in parallel. This is particular advantageous for compound screening, since several dilutions of the test compounds have to be tested and various standard compounds in order to compare them as a profile with those of the test compounds are usually employed in such screening methods. Thus, the method of the present invention for first time enables the use of embryoid bodies for compound screening in a cost-effective manner and on a reasonable industrial scale.

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In some embodiments of the present invention, further steps can be performed such as dividing the cell aggregates obtained in step (b) to the desired final concentration, for example for use in toxicity tests and/or for the production of certain cell types and tissue.

- 5 In the absence of feeder cells, exogenous leukemia inhibitory factor (LIF), or conditioned medium, ES or EG cells in the form of embryoid bodies spontaneously differentiate into a wide variety of cell types, including cells found in each of the endoderm, mesoderm, and ectoderm germ layers. With the appropriate combinations of growth and differentiation factors, however, cell differentiation can be controlled. For example, EBs cells can generate
- 10 cells of the hematopoietic lineage in vitro (Keller et al., *Mol. Cell. Biol.* 13 (1993), 473-486; Palacios et al., *Proc. Natl. Acad. Sci USA* 92 (1995), 7530-7534; Rich, *Blood* 86 (1995), 463-472). Additionally, mouse ES cells have been used to generate in vitro cultures of neurons (Bain et al., *Developmental Biology* 168 (1995), 342-357; Fraichard et al., *J. Cell Science* 108 (1995), 3161-3188), cardiomyocytes (heart muscle cells) (Klug et al., *Am. J. Physiol.* 269 (1995), H1913-H1921), skeletal muscle cells (Rohwedel et al., *Dev. Biol.* 164 (1994), 87-101), vascular cells (Wang et al., *Development* 114 (1992), 303-316), US patent US-A-5,773,255 relates to glucose-responsive insulin secreting pancreatic beta cell lines, US patent US-A-5,789,246 relates to hepatocyte precursor cells. Hepatic differentiation of murine embryonic stem cells is also described in Jones et al., *Exp. Cell Res.* 272 (2002), 15-22.
- 20 Other progenitors of interest include but are not limited to chondrocytes, osteoblasts, retinal pigment epithelial cells, fibroblasts, skin cells such as keratinocytes, dendritic cells, hair follicle cells, renal duct epithelial cells, smooth and skeletal muscle cells, testicular progenitors, and vascular endothelial cells. Embryonic stem cell differentiation models for cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell
- 25 differentiation in vitro have been generally described in Guan et al., *Cytotechnology* 30 (1999), 211-226.

In certain embodiments of the invention, differentiation is promoted by withdrawing one or more medium component(s) that promote(s) growth of undifferentiated cells, or act(s) as an inhibitor of differentiation. Examples of such components include certain growth factors, mitogens, leukocyte inhibitory factor (LIF), and basic fibroblast growth factor (bFGF). Differentiation may also be promoted by adding a medium component that promotes differentiation towards the desired cell lineage, or inhibits the growth of cells with undesired characteristics.

Hence, in a further embodiment the method of the present invention further comprises culturing the cells and cell aggregates, i.e. embryoid bodies, respectively, under conditions allowing differentiation of the cells into at least one cell type such as those mentioned above.

5 Of course, the multi- or pluripotent cells used for the production for the embryoid bodies in accordance with the method of the present invention may not be native but genetically engineered, for example with reporter gene constructs and/or other transgenes, for example such of which the function in cell development and differentiation is desired to be elucidated. Furthermore, as mentioned above, in accordance with this invention, embryoid bodies
10 obtained by the above described methods can be induced to develop particular cell types and tissue. Populations of differentiated cells can be depleted of relatively undifferentiated cells and/or of cells of undesired cell types by using a selection system that is lethal to the undesired cells and cell types, i.e. by expressing a selectable marker gene that renders cells of a specific cell type resistant to a lethal effect of an external agent, under control of a
15 regulatory sequence that causes the gene to be preferentially expressed in the desired cell type and/or at a certain stage of development. To accomplish this, the cells are genetically altered before the process used to differentiate the cells into the desired lineage for therapy, in a way that the cells comprises a selectable marker operably linked to a cell type specific regulatory sequence specific for the desired cell type.

20 Any suitable expression vector for this purpose can be used. Suitable viral vector systems for producing stem cells altered according to this invention can be prepared using commercially available virus components. The introduction of the vector construct or constructs into the embryonic stem cells occurs in a known manner, e.g. by transfection, electroporation,
25 lipofection or with the help of viral vectors. Viral vectors comprising effector genes are generally described in the publications referenced in the last section. Alternatively, vector plasmids can be introduced into cells by electroporation, or using lipid/DNA complexes. Exemplary is the formulation Lipofectamine 2000(TM), available from Gibco/Life Technologies. Another exemplary reagent is FuGENE(TM) 6 Transfection Reagent, a blend
30 of lipids in non-liposomal form and other compounds in 80% ethanol, obtainable from Roche Diagnostics Corporation. Preferably, the vector constructs and transfection methods described in WO02/051987 are used; the disclosure content of which is incorporated herein by reference.

Resistance genes per se are known. Examples for these are nucleoside and aminoglycoside-antibiotic-resistance genes conferring resistance to, e.g. puromycin, neomycin, or hygromycin. Further examples for resistance genes are dehydrofolate-reductase, which confers a resistance against aminopterin and methotrexate, as well as multi drug resistance genes, which confer a resistance against a number of antibiotics, e.g. against vinblastin, doxorubicin and actinomycin D.

In a particularly preferred embodiment of the present invention, said selectable marker confers resistance to puromycin. Puromycin is particularly suited for the fast elimination of non-cardiac cells in adherent culture of transgenic EBs. Furthermore, drug selection of cardiac cells can be implemented entirely in the suspension culture of transgenic EBs. Hence, it could also be shown that purified ES derived cardiomyocytes survive much longer in culture than untreated counterparts. Moreover, the elimination of undifferentiated ES cells during drug selection process has itself been shown to have clear positive effect on viability and longevity of such differentiated ES derived cells as cardiomyocytes. In addition, it could be surprisingly shown that the release from surrounding non-differentiated cells induces proliferation of cardiomyocytes. Thus, the drug selection possesses both purifying and multiplying effect.

In a preferred embodiment of the invention, said multi- or pluripotent cells of said EBs comprise a reporter gene, preferably wherein said reporter is operably linked to a cell type specific regulatory sequence specific for a certain cell type. This type of vector has the advantages of providing visualization of differentiation, definition of the time point for beginning of drug selection, visualization of drug selection and tracing of the fate of purified cells grafted in recipient tissue. Such vectors, which are preferably employed in accordance with the methods of the present invention are described in WO02/051987. Usually, said cell type specific regulatory sequence of the reporter gene is substantially the same as said cell type specific regulatory sequence of the marker gene. This can advantageously be achieved by putting said marker gene and said reporter gene into the same recombinant nucleic acid molecule, i.e. vector used for stem cell transfection, preferably such that said marker gene and said reporter gene are contained on the same cistron.

The reporter can be of any kind as long as it is non-damaging for the cell and confers an observable or measurable phenotype. According to the present invention, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (described in WO95/07463, WO96/27675 and WO95121 191) and its derivatives "Blue GFP" (Heim et al., Curr. Biol. 6 (1996), 178-182 and Redshift GFP" (Muldoon et al., Biotechniques 22 (1997), 162-167) can

be used. Particularly preferred is the Enhanced Green Fluorescent Protein (EGFP). Further embodiments are the Enhanced Yellow and Cyan Fluorescent Proteins (EYFP and ECFP, respectively) and Red Fluorescent proteins (DsRed, HcRed). Further fluorescent proteins are known to the person skilled in the art and can be used according to the invention as long as they do not damage the cells. The detection of fluorescent proteins takes place through per se known fluorescence detection methods; see, e.g., Kolossov et al., J. Cell Biol. 143 (1998), 2045-2056. Alternatively to the fluorescent proteins, particularly in in vivo applications, other detectable proteins, particularly epitopes of those proteins, can also be used. Also the epitope of proteins, though able to damage the cell per se, but whose epitopes do not damage the cells, can be used; see also WO02/051987.

For the selection for stably transfected ES cells vector constructs contain a further selectable marker gene, which confers e.g. a resistance against an antibiotic, e.g. neomycin. Of course, other known resistance genes can be used as well, e.g. the resistance genes described above in association with the fluorescent protein encoding genes. The selection gene for the selection for stably transfected ES-cells is under the control of a different promoter than that which regulates the control of the expression of the detectable protein. Often constitutively active promoters are used, e.g. the PGK-promoter.

The use of a second selection gene is advantageous for the ability to identify the successfully transfected clones (efficiency is relatively low) at all. Otherwise a smothering majority of non-transfected ES-cell may exist and during differentiation, e.g., no EGFP positive cells might be detected.

In a further embodiment of the invention the cells can be manipulated additionally so that specific tissues are not formed. This can occur for instance by inserting of repressor elements, e.g. a doxizyclin inducible repressor element. Thereby, a possible contamination of the desired differentiated cells with pluripotent, potentially tumourigenic cells can also be excluded.

The desired cell type intended for the stem cells and embryoid bodies to differentiate to may be of any kind and includes but not limited to neuronal cells, glial cells, cardiomyocytes, glucose-responsive insulin secreting pancreatic beta cells, hepatocytes, astrocytes, oligodendrocytes, chondrocytes, osteoblasts, retinal pigment epithelial cells, fibroblasts, keratinocytes, dendritic cells, hair follicle cells, renal duct epithelial cells, vascular endothelial cells, testicular progenitors, smooth and skeletal muscle cells; see also supra.

In a particular preferred embodiment of the invention, said cell type are cardiomyocytes. For this embodiment, a cell type specific regulatory sequence for driving a drug resistance gene is used, which is preferably atrial and/or ventricular specific. Corresponding regulatory sequences, i.e. cardiac specific promoters are described for Nkx-2.5 specific for very early cardiomyocytes and mesodermal precursor cells respectively, (Lints et al., Development 119 (1993), 419-431); human-cardiac- α -actin specific for heart tissue, (Sartorelli et al., Genes Dev. 4 (1990), 1811-1822), and MLC-2V specific for ventricular heart muscle cells (O'Brien et al., Proc. Natl. Acad. Sci. U.S.A. 90 (1993), 5157-5161 and WO-A-96/16163). Cardiac specific alpha-myosin heavy chain promoter is described in Palermo et al., Cell Mol Biol Res 41 (1995), 501-519; Gulick et al., J. Biol. Chem. 266 (1991), 9180-91855; the myosin light chain-2v (MLC2v) promoter also by Lee et al., Mol. Cell Biol. 14 (1994), 1220-1229; Franz et al., Circ Res 73 (1993), 629-638; see also expression of the atrial specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart described in Yutzey et al., Development 120 (1994), 871-883.

The present invention also relates to embryoid bodies and differentiated cells and tissue derived from said embryoid bodies. Hence, said cells are preferably embryonic cell type and/tissue specific cells, most preferably cardiac tissue. Likewise, organ constituted from those cells, cell aggregates and tissue are subject of the present invention as well as implants or transplants comprising such cells, cell aggregates, tissue or organ. All of those can be used in a method of treatment of damaged tissue or organs in a subject comprising implanting or transplanting to the subject in need thereof. Hence, compositions, such as pharmaceutical compositions comprising any one of those cell aggregates, or tissue of the present invention as described herein are encompassed in the scope of the present invention. As described before, those compositions and methods of the invention can be used for a variety of purposes, for example for analyzing early steps of tissue formation during embryonic development or the influence of factors and compounds on this process. Furthermore, the EBs can be used for the preparation of transgenic non-human animal animals. The generation of transgenic animals from ES cells is known in the art; see, e.g., A. L. Joyner Ed., Gene Targeting, A Practical Approach (1993), Oxford University Press. A general method for making transgenic non-human animals is described in the art, see for example WO94/24274.

Hence, the present invention generally relates to the use of the afore-described method of the present invention, the embryoid bodies obtained thereby as well as differentiated cells and tissue thereof for loss of function assays of specific genes, gain of function assays of exogenous genes, developmental analysis of teratogenic/embryotoxic compounds, pharmacological assays, microarray systems, establishment of model systems for pathological cell functions, application of differentiation and growth factors for induction of selectively differentiated cells or as a source for tissue grafts. In one embodiment, the fate of the cell types and formation of cell aggregates and tissue as well physiological and/or developmental status of the cells or cell aggregate are analyzed, for example by isometric tension measurements, echocardiography and the like. Preferably, the status of the cells or cell aggregates is analyzed by monitoring the differentiation of electrical activity of the cells on an array, for example by recording the extracellular field potentials with a microelectrode arrays (MEA). For example, electrophysiological properties during the ongoing differentiation process of embryonic stem cells differentiating into cardiac myocytes can be followed by recordings of extracellular field potentials with microelectrode arrays (MEA) consisting of, e.g., 60 substrate-integrated electrodes; see Banach et al. *Am. J. Physiol. Heart Circ. Physiol.* (2003), Feb 6, p S0363-6135. Multiple arrays of tungsten microelectrodes were used to record the concurrent responses of brain stem neurons that contribute to respiratory motor pattern generation; see Morris et al., *Respir. Physiol.* 121 (2000), 119-133.

The EBs and methods of the present invention are particularly suited for use in drug screening and therapeutic applications. For example, differentiated EBs of this invention can be used to screen for substances (such as solvents, small molecule drugs, peptides, polynucleotides, and the like), particularly household products, see *supra*, or environmental conditions (such as culture conditions or manipulation) that affect the characteristics of differentiated cells. Particular screening applications of this invention relate to the testing of pharmaceutical compounds in drug research. The reader is referred generally to the standard textbook "In vitro Methods in Pharmaceutical Research", Academic Press, 1997, and US patent US-A-5,030,015).

The present invention also relates to kit compositions containing specific reagents such as those described herein-before useful for conducting any one of the above described methods of the present invention, containing for example culture media components, selectable markers, reference samples, microarrays, vectors, probes, containers, multi- or pluripotent

cells. Such a kit would typically comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents useful for performing said methods. The carrier may also contain a means for detection such as labeled enzyme substrates or the like. Hence, the present invention also relates to the use of cell containers, devices for agitation and/or culturing cells, culture media and components thereof, multi- or pluripotent cells, vectors, and microarrays for a method of the invention described hereinbefore.

Hence, the means and methods of the present invention described herein-before can be used in a variety of applications including but not limited to "loss of function" assays with ES cells containing homozygous mutations of specific genes, "gain of function" assays with ES cells overexpressing exogenous genes, developmental analysis of teratogenic/embryotoxic compounds in vitro, pharmacological assays and the establishment of model systems for pathological cell functions, and application of differentiation and growth factors for induction of selectively differentiated cells which, can be used as a source for tissue grafts; see for review, e.g., Guan et al., *Altex* 16 (1999), 135-141.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the materials, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for example electronic devices. For example the public database "Medline" may be utilized, which is hosted by the National Center for Biotechnology Information and/or the National Library of Medicine at the National Institutes of Health. Further databases and web addresses, such as those of the European Bioinformatics Institute (EBI), which is part of the European Molecular Biology Laboratory (EMBL) are known to the person skilled in the art and can also be obtained using internet search engines. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, *TIBTECH* 12 (1994), 352-364.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention. The contents of all cited references (including literature references, issued patents,

published patent applications as cited throughout this application and manufacturer's specifications, instructions, etc) are hereby expressly incorporated by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

5 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art.

For further elaboration of general techniques concerning stem cell technology, the practitioner can refer to standard textbooks and reviews, for example Teratocarcinomas and embryonic
 10 stem cells: A practical approach (E. J. Robertson, ed., IRL Press Ltd. 1987); Guide to Techniques in Mouse Development (P. M. Wasserman et al., eds., Academic Press 1993); Embryonic Stem Cell Differentiation in Vitro (Wiles, Meth. Enzymol. 225 (1993), 900,); Properties and uses of Embryonic Stem Cells: Prospects for Application to Human Biology and Gene Therapy (Rathjen et al., Reprod. Fertil. Dev. 10 (1998), 31,). Differentiation of stem
 15 cells is reviewed in Robertson, Meth. Cell Biol. 75 (1997), 173; and Pedersen, Reprod. Fertil. Dev. 10 (1998), 31. Besides the sources for stem cells described already above further references are provided; see Evans and Kaufman, Nature 292 (1981), 154-156; Handyside et al., Roux's Arch. Dev. Biol., 196 (1987), 185-190; Flechon et al., J. Reprod. Fertil. Abstract Series 6 (1990), 25; Doetschman et al., Dev. Biol. 127 (1988), 224-227; Evans et al.,
 20 Theriogenology 33 (1990), 125-128; Notarianni et al., J. Reprod. Fertil. Suppl., 43 (1991), 255-260; Giles et al., Biol. Reprod. 44 (Suppl. 1) (1991), 57; Strelchenko et al., Theriogenology 35 (1991), 274; Sukoyan et al., Mol. Reprod. Dev. 93 (1992), 418-431; Iannaccone et al., Dev. Biol. 163 (1994), 288-292.

25 Methods in molecular genetics and genetic engineering are described generally in the current editions of Molecular Cloning: A Laboratory Manual, (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984);
 30 Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Gene Transfer Vectors for Mammalian Cells (Miller & Calos, eds.); Current Protocols in Molecular Biology and Short Protocols in Molecular Biology, 3rd Edition (F. M. Ausubel et al., eds.); and Recombinant DNA Methodology (R. Wu ed., Academic Press). Gene Transfer Vectors For Mammalian Cells (J.

H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986). Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, and Clontech. General techniques in cell culture and media collection are outlined in Large Scale Mammalian Cell Culture (Hu et al., Curr. Opin. Biotechnol. 8 (1997), 148); Serum-free Media (Kitano, Biotechnology 17 (1991), 73); Large Scale Mammalian Cell Culture (Curr. Opin. Biotechnol. 2 (1991), 375); and Suspension Culture of Mammalian Cells (Birch et al., Bioprocess Technol. 19 (1990), 251). Other observations about the media and their impact on the culture environment have been made by Marshall McLuhan and Fred Allen.

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EXAMPLES

Example 1: Culture of ES cells on mouse embryonic feeder cells

ES cells (e.g. clone D3, ATCC, CRL 1934) were cultured on 10cm petri dishes (Falcon, Becton Dickinson) at a density of 1.4×10^6 in DMEM (Gibco, Invitrogen) supplemented with 15% FCS (Gibco, invitrogen, batch controlled) and 1×10^3 U/ml LIF (Chemicon) on a layer of feeder cells (inactivated mouse embryonic fibroblasts, prepared according standard protocols; see also description of the invention above). Cells were incubated at 37°C, 7% CO₂ and 95% humidity. Cells were split every second day by trypsinizing them to single cell suspension and seeding 1.4×10^6 on a fresh 10cm dish coated with feeder cells.

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Example 2: ES cell aggregation and preparation of Embryoid Bodies

ES cells from one ore more petri dishes were trypsinised to obtain a single cell suspension and collected by centrifugation (800g for 5 min). Cells were resuspended to a density of 2×10^6 cells/ml in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 20% (v/v) fetal bovine serum (FBS, Invitrogen, batch controlled). 4ml of this suspension were incubated per 6 cm petri dish (bacterial grade, Greiner) on a rocking table (GFL, model

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3006) at 50 rpm, 37°C, 5% CO₂ and 95% humidity for 6h. After this time, the suspension was diluted 1:10 (e.g. 2ml of suspension added to 18ml IMDM 20% FCS) in several T25 tissue culture flasks (Falcon, Becton Dickinson) and incubated under the same conditions for additional 18h. After this time, ES cell aggregates ("embryoid bodies", EBs) of homogeneous shape and size are formed, typically around 500 per ml of suspension.

Example 3: ES cell differentiation

As an example, EBs raised by the method described above can be differentiated to cardiac cells in 96 well plates. EBs were collected from the flasks into a 10cm bacterial petri dish and individually picked into the wells of a 96 well flat bottom microtiter plate (Falcon, Becton Dickinson) coated with 0.2% gelatine. EBs were incubated in 200µl of IMDM 20% FCS at 37°C, 5% CO₂, 95% humidity. Half of the medium was exchanged twice a week. After 7-10 days, first beating cardiomyocytes appeared in the EBs and could be visualized using an appropriate microscope.

Adding of compounds interfering with normal cardiac physiology (e.g. Nifedipine, 4-aminopyridine) alter beating frequency and/or intensity. Therefore, this method can be used in pharmacological compound screening.

Additionally, the method can be used to detect embryotoxic compounds in an high-throughput in vitro system. Therefore, the EBs can be manufactured using ES cells with an appropriate reporter gene (e.g. a fluorescent reporter like GFP) driven by an tissue specific promoter (e.g. α-MHC for cardiomyocytes). After plating into 96 well plates (flat bottom, black; Falcon, Becton Dickinson), the EBs are challenged with the test compounds at different concentrations or with the diluents as control. Half of the medium is replaced with fresh medium and compound twice a week. After differentiation towards cardiomyocytes appears in the control EBs, the fluorescence in all EBs is measured using a fluorescence spectrophotometer (Tecan). The embryotoxic effect of the test compounds is calculated as percent of the controls, which are defined as 100%.

Claims

1. A method for producing embryoid bodies (EBs) from multi- or pluripotent cells comprising
 - (c) agitation of a liquid suspension culture of multi- or pluripotent cells in a container at a concentration of about 1 to 5×10^6 cells/ml until generation of cell aggregates; and
 - (d) diluting the suspension and agitation of the suspension until formation of EBs.
2. The method of claim 1, wherein prior to step (a) the cells are cultured on embryonic mouse fibroblasts (feeder cells).
3. The method of claim 1 or 2, wherein said multi- or pluripotent cells are embryonic stem (ES) cells, primordial germ (EG) cells or adult stem cells.
4. The method of any one of claims 1 to 3; wherein the culture medium in step (a) and/or (b) is IMDM 20% FCS and 5% CO₂.
5. The method of any one of claims 1 to 4, wherein the culture conditions in step (a) and/or (b) comprise 37°C and 95% humidity.
6. The method of any one of claims 1 to 5, wherein the suspension in step (a) is cultured for about 6 hours and/or in step (b) for about 16 to 20 hours.
7. The method of any one of claims 1 to 6, wherein the incubation step is conducted in a container made of plastic.
8. The method of any one of claims 1 to 7, wherein the suspension culture in step (a) is present in a petri dish and/or in step (b) in a culture flask, beaker or tumbler.
9. The method of claim 8, wherein the suspension in step (b) is cultured in T25 flasks.
10. The method of any one of claims 1 to 9, wherein said dilution in step (b) is 1:10.

11. The method of any one of claims 1 to 10, wherein said agitation in step (a) and/or (b) is a horizontal agitation.
12. The method of any one of claims 1 to 11, wherein the final concentration of EBs in the suspension culture is about 500/ml.
13. The method of any one of claims 1 to 12, further comprising dividing the cell aggregates to the desired final concentration.
14. The method of any one of claims 1 to 13, further comprising culturing the cells under conditions allowing differentiation of the cells into at least one cell type.
15. The method of claim 14, wherein said cell type is selected from cardiomyocytes, neurons, endothelial cells, fibroblasts, skeletal muscle cells, smooth muscle cells and chondrocytes.
16. The method of any one of claims 1 to 15, further comprising selection of desired cell types by use of one or more selectable markers and/or agents.
17. The method of any one of claims 1 to 16, wherein said cell are genetically engineered.
18. The method of any one of claims 1 to 17, wherein said cell comprise a selectable marker and/or a reporter gene.
19. The method of any one of claims 1 to 18, wherein the cells are embryonic stem cells.
20. An embryoid body obtained by the method of any one of claims 1 to 19.
21. A differentiated cell or tissue derived from the embryoid body of claim 20.
22. The differentiated cell of claim 21, wherein the cell is a cardiomyocyte.
23. Use of the method of any one of claims 1 to 19, the embryoid body of claim 20 or the cell or tissue of claim 21 or 22 for loss of function assays of specific genes, gain of function assays of exogenous genes, developmental analysis of teratogenic/embryotoxic

compounds, pharmacological assays, microarray systems, establishment of model systems for pathological cell functions, application of differentiation and growth factors for induction of selectively differentiated cells or as a source for tissue grafts.

- 5 24. Kit for use in a method of any one of claims 1 to 19 comprising culture media components, selectable markers, reference samples, microarrays, vectors, probes, containers, multi- or pluripotent cells.
- 10 25. Use of cell container, devices for agitation and/or culturing cells, culture media and components thereof, multi- or pluripotent cells, vectors, or microarray for a method of any one of claims 1 to 19.

ABSTRACT

Provided are means and methods for producing embryoid bodies (EBs) from multi- or pluripotent cells. In particular, a method of generating embryoid bodies (EBs) is described comprising agitation of a liquid suspension culture of multi- or pluripotent cells in a container at a concentration of about 1 to 5×10^6 cells/ml until generation of cell aggregates, diluting the suspension and agitation of the suspension until formation of EBs. Furthermore, the present invention relates to the use of the novel method and EBs obtained thereby for a variety of applications including genomics, diagnostic assays, teratogenic/embryotoxicological and pharmacological assays as well as for the provision of tissue grafts.